Possible Dissociation of the Heparin-binding and Mitogenic Activities of Heparin-binding (Acidic Fibroblast) Growth Factor-1 from Its Receptor-binding Activities by Site-directed Mutagenesis of a Single Lysine Residue

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Abstract. The fibroblast or heparin-binding growth factors (HBGFs) are thought to be modulators of cell growth and migration, angiogenesis, wound repair, neurite extension, and mesoderm induction. A better understanding of the structural basis for the different activities of these proteins should facilitate the development of agonists and antagonists of specific HBGF activities and identification of the signal transduction pathways involved in the mechanisms of action of these growth factors. Chemical modification studies of Harper and Lobb (Harper, J. W., and R. R. Lobb. 1988. *Biochemistry.* 27:671–678) implicated lysine 132 in HBGF-1 (acidic fibroblast growth factor) as being important to the heparin-binding, receptor-binding, and mitogenic activities of the protein. We changed lysine 132 to a glutamic acid residue by site-directed mutagenesis of the human cDNA and expressed the mutant protein in *Escherichia coli* to obtain sufficient quantities for functional studies. Replacement of this lysine with glutamic acid reduces the apparent affinity of HBGF-1 for immobilized heparin (elutes at 0.45 M NaCl vs. 1.1 M NaCl for wild-type). Mitogenic assays established two points: (*a*) human recombinant HBGF-1 is highly dependent on the presence of heparin for optimal mitogenic activity, and (*b*) the change of lysine 132 to glutamic acid drastically reduces the specific mitogenic activity of HBGF-1. The poor mitogenic activity of the mutant protein does not appear to be due to a reduced affinity for the HBGF receptor. Similarly, the mutant HBGF-1 can stimulate tyrosine kinase activity and induce protooncogene expression. Differences in the biological properties of the wild-type and mutant proteins were observed in transfection studies. Mutant HBGF-1 expression in transfected NIH 3T3 cells did not induce the same transformed phenotype characteristic of cells expressing wild-type HBGF-1. Together these data indicate that different functional properties of HBGF-1 may be dissociated at the structural level.

THE heparin-binding growth factor (HBGF)[1] family presently consists of seven structurally related polypeptides (3). The cDNAs for each have been cloned and sequenced. Two of the proteins, HBGF-1 and HBGF-2, have been characterized under many different names, but most often as acidic and basic fibroblast growth factor, respectively. Three sequence-related oncogenes have been identified; the hst oncogene was discovered based on its ability to transform NIH 3T3 cells (9, 25, 38, 45); the int-2 oncogene was first identified as a gene activated by mouse mammary tumor virus (7, 10, 11) and the FGF-5 oncogene was identified using NIH 3T3 transformation assays (46, 47). Recently a gene termed FGF-6 was identified by screening a mouse cosmid library with a human hst probe under reduced stringency and was shown to be capable of transforming NIH 3T3 cells (32). Finally, an epithelial cell-specific growth factor termed KGF or FGF-7 has been identified and its cDNA cloned and sequenced (13).

Functions associated with HBGF-1 and HBGF-2 include stimulation of mitogenesis, chemotaxis, mesoderm induction, neurite extension, and plasminogen activator activity. These HBGFs also induce angiogenesis in vivo and accelerate wound repair (for reviews see references 3, 18, 27, 36). The mechanisms by which HBGFs promote these functions are poorly understood but may include activation of protein tyrosine kinase activity (8, 15, 20), phosphorylation of phospholipase C-γ (6), and activation of immediate-early gene transcription (17). In addition, both HBGF-1 and HBGF-2 have been shown to be relatively resistant to degradation after internalization by receptor-mediated endocytosis (14, 24,

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34). Intact growth factor persists intracellularly for several hours and large fragments (15,000 and 10,000 M_r for HBGF-1; 16,000 M_r for HBGF-2) are detectable after as many as 24 h. Further, nuclear or nucleolar localization of HBGF-2 has been observed (2, 35).

Despite the identification of additional members of the HBGF family and a broad range of cells and tissues that contain the growth factors, and despite the availability of large quantities of recombinant protein and increased knowledge of the broad spectrum of activities of potential biological significance that can be attributed to the HBGFs, relatively little is known regarding the relationship of these highly conserved structures to any of their known functions. Baird et al. (1) reported the synthesis of 25 peptides, which together encompass and overlap the entire sequence of HBGF-2 as described by Ueno et al. (42). They reported the identification of two functional domains in the primary structure of HBGF-2 based on the abilities of synthetic peptides to interact with HBGF receptor, bind radiolabeled heparin in a solid phase assay, and inhibit HBGF-2 stimulation of thymidine incorporation into DNA. Using the numbering system of the authors (which does not correspond to full length HBGF-2) statistically significant functional activities could be assigned to peptides corresponding to residues 24–68 and 105–115 of HBGF-2. Similarly, Schubert et al. (39) demonstrated that peptides corresponding to residues 1–24, 24–68, and 93–120 of HBGF-2 are able to stimulate substratum adhesion of PC12 cells. We have shown that a synthetic peptide corresponding to residues 49–72 of HBGF-1 (using numbering of 1–154 for full length HBGF-1) is able to compete with HBGF-1 for heparin binding in a gel overlay assay (33). This region is homologous to one of the regions of HBGF-2 (residues 24–68) described above as possessing heparin-binding activity.

To date, the most complete and informative studies documenting the effects of chemical modification of any HBGF on function are those of Harper and Lobb (19). Briefly, they were able to show that limited reductive methylation of bovine HBGF-1 with formaldehyde and cyanoborohydride resulted in stoichiometric methylation only of lysine 132 (using 1–154 numbering for full length HBGF-1). They reported 90% modification of this residue, with 60% dimethyllysine. The modified protein exhibited significantly reduced apparent affinity for immobilized heparin (eluted at ~0.7 M NaCl vs. ~1.2 M NaCl for unmodified HBGF-1), a fourfold reduction in its ability to stimulate DNA synthesis in NIH 3T3 fibroblasts and a similar reduction in its ability to compete with labeled ligand in a radioreceptor assay. A lysine residue is found at this position of HBGF-1 and HBGF-2 of all species characterized to date. Together these data implicate a crucial role for lysine 132 in several of the known functions of HBGF-1.

In this report we address the role of lysine 132 in HBGF-1 function using site-directed mutagenesis of this position to a glutamic acid. This approach offers several advantages over chemical modification studies including (*a*) the ability to produce large quantities of the desired product, (*b*) elimination of significant (although sub-stoichiometric) modification of other lysines, and (*c*) allowing the introduction of modified HBGF-1 into mammalian cells through transfection of cDNA expression vectors designed to produce the desired mutant. Despite these advantages the importance of chemical modification studies such as those of Harper and Lobb (19) should not be underestimated for they are extremely useful in the design of a rational approach to site-directed mutagenesis. The results described here demonstrate that replacement of lysine 132 of HBGF-1 with glutamic acid reduces significantly its apparent affinity for immobilized heparin and its mitogenic capacity. However, the apparent affinity of the mutant for high affinity cell surface receptors appears unaltered. When assayed in the presence of heparin where the difference in wild-type and mutant HBGF-1 mitogenic activity is most apparent, mutant HBGF-1 can stimulate tyrosine kinase activity and induce protooncogene expression. Functional differences between the wild-type and mutant HBGF-1 are also apparent after transfection of cDNA expression vectors into NIH 3T3 fibroblasts.

Materials and Methods

Materials

Heparin-Sepharose, protein A–Sepharose, pKK233 expression vectors, and low molecular weight markers were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). All reagents for PAGE and the Mighty Small Apparatus were from Hoefer Scientific Instruments (San Francisco, CA). Reagents for reversed-phase HPLC, amino acid analysis, and amino acid sequencing were purchased from Applied Biosystems, Inc. (Foster City, CA). Isotopes and the in vitro mutagenesis system were from Amersham Corp. (Arlington Heights, IL). The rabbit polyclonal HBGF-1–specific antibody was provided by R. Friesel (American Red Cross, Rockville, MD) and the rabbit polyclonal anti–phospholipase C-γ antibodies were provided by A. Zilberstein (Rorer Biotechnology, Inc., King of Prussia, PA). Tissue culture media and plasticware were purchased from Gibco Laboratories (Grand Island, NY). High molecular weight molecular markers were from Bio-Rad Laboratories (Richmond, CA). Endoproteinase ASP-N and the random primer DNA labeling kit were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Other chemicals were reagent grade.

Construction of pREC and pI32E Prokaryotic Expression Plasmids

The plasmid expressing wild-type HBGF-1 (corresponding to the α-form of endothelial cell growth factor (5), pREC, was kindly provided by R. Forough (American Red Cross). This plasmid was constructed by cloning synthetic oligonucleotide cassettes into the Nco I/Hind III site of pKK233-2. The plasmid expressing mutant HBGF-1 (glutamic acid instead of lysine at amino acid position 132; pI32E) was constructed as follows. The Eco RI/Hind III fragment of HBGF-1 cDNA clone 1 (21) was subcloned into M13mp18. Single-stranded template was prepared and used for oligonucleotide-directed in vitro mutagenesis. Double-stranded DNA was transformed into *E. coli* TG-1 cells and the resultant plaques were screened by M13 dideoxy sequencing. The mutated HBGF-1 cDNA was transferred into the expression vector pKK223-3 using the original Eco RI and Hind III sites.

Production and Purification of Recombinant Proteins

Recombinant plasmids pREC or pI32E were introduced into the *lacI*^{q}-bearing *Escherichia coli* strain JM103. Cultures of JM103 bearing the recombinant plasmids were grown with shaking at 37°C in Luria broth containing 100 μg/ml ampicillin. A fresh overnight culture was diluted and grown until the A_{550} reached ~0.2, at which point isopropylthio-β-galactoside was added to 1 mM. Cells were collected by centrifugation and frozen at −80°C for subsequent growth factor purification.

The frozen cell pellets from 2-liter cultures were resuspended in 50 ml of 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 50 mM glucose. A fresh solution of hen egg lysozyme in the same buffer was added to 10 μg/ml. The cells were mixed at 4°C for 45 min. The viscous lysate was sonicated at maximum intensity using a large probe and four 20-s pulses of a Heat Systems W-380 sonicator. The lysate was clarified by centrifugation at 6,000 *g* for 15 min at 4°C. The supernatant was diluted to 100 ml with 50 mM Tris-HCl, pH 7.5, 10 mM EDTA and incubated with 20 ml of hydrated heparin-

Sepharose at 4°C with end-over-end mixing for 2 h. The resin was eluted batchwise using a sintered glass funnel and successive washes of the same buffer containing 0, 0.1, 0.5, 0.65, and 1.5 M NaCl.

The wild-type recombinant HBGF-1 eluted with the 1.5 M NaCl wash. The mutant was eluted with the 0.5 M NaCl wash. Although the wild-type protein was essentially pure after heparin-Sepharose chromatography, the mutant HBGF-1 constituted only 10–20% of the 0.5 M NaCl wash. Both preparations were purified to >95% purity using reversed-phase HPLC (4). The reversed-phase purified material was used for all reported studies.

Characterization of Recombinant Proteins

All preparations of purified recombinant human wild-type and mutant HBGF-1 were analyzed by SDS-PAGE, amino acid analysis, amino terminal sequencing, peptide mapping, and amino acid sequencing of the peptide encompassing the mutated residue. Protein concentrations were determined by amino acid analysis. Aliquots of wild-type and mutant HBGF-1 were subjected to electrophoresis using the SDS-PAGE system of Laemmli (26). A 15% acrylamide, 0.4% *N,N*-methylenebisacrylamide solution was polymerized in a Hoefer mini-gel apparatus and electrophoresis was carried out at a constant 200 V. Protein was visualized by staining the gel with 0.1% Coomassie blue R-250 in 50% methanol, 10% glacial acetic acid, and destaining with 9% glacial acetic acid, 5% methanol. Samples for amino acid analysis were hydrolyzed with argon-purged, constant boiling 6 N HCl at 115°C for 18 h using a Pico-Tag workstation (Waters Associates, Milford, MA). Amino acids were derivatized with phenylisothiocyanate and separated with a PTC analyzer (model 130A; Applied Biosystems, Inc.). A Waters 840 system was used for data collection and reduction. Amino acid sequences were established using a protein sequencer (model 477A; Applied Biosystems, Inc.) using modified Edman chemistry and an on-line model 120A PTH analyzer. Peptide mapping of recombinant protein after digestion with endoproteinase Asp-N at a 1:25 ratio of enzyme to protein in 50 mM Na_2HPO_4, pH 8.0, 37°C for 18 h was performed using a microbore HPLC system (model 130A; Applied Biosystems, Inc.). The appropriate peptides were subjected to amino acid sequence analysis to establish the fidelity of expression of the wild-type and mutant HBGF-1 vectors.

Stability Studies

Metabolically labeled recombinant proteins were prepared by growing bacterial cultures as described above until the A_{550} reached ~0.4, at which point the cells were collected by centrifugation. They were resuspended in 98.5% M9 minimal medium/1.5% Luria broth and [^{3}H]leucine (140 Ci/mmol) was added to 45 μCi/ml. Cells were grown with shaking for 30 min, and then for an additional 4 h in the presence of 1 mM isopropylthio-β-galactoside. Cells were collected and growth factors purified as described above. The purified, labeled growth factors were incubated for 48 h at 37°C in the presence of media (DMEM containing 10% calf serum) that had been conditioned for 48 h by NIH 3T3 cells. The growth factor–containing media was analyzed by SDS-PAGE and autoradiography.

Mitogenic Assays

The mitogenic activities of wild-type and mutant recombinant HBGF-1 were determined by measuring their ability to stimulate DNA synthesis in NIH 3T3 cells and to support the proliferation of human umbilical vein endothelial cells. DNA synthesis was determined by measuring the amount of [^{3}H]thymidine incorporated into cells. Briefly, NIH 3T3 cells were seeded into 48-well plates and grown to near confluence in DME containing 10% calf serum. The cells were serum starved (DME, 0.5% calf serum) for 24 h. Mitogens were added to the wells and incubated for 18 h. The cells were pulsed with 0.5 μCi/ml of [^{3}H]thymidine (25 Ci/mmol) for 4 h. The cells were rinsed with PBS, fixed with 10% TCA, rinsed with PBS, and then solubilized with 0.5 N NaOH. Incorporation of [^{3}H]thymidine into acid-insoluble material was determined by scintillation counting. All assays were performed in triplicate.

Human umbilical vein endothelial cells were provided by T. Maciag (American Red Cross, Rockville, MD). They were maintained on fibronectin-coated plates (2 μg/cm^2) in medium 199 supplemented with 10% (vol/vol) heat-inactivated FBS, 1× antibiotic-antimycotic, 10 U/ml heparin, and 10 ng/ml human recombinant HBGF-1. For growth assays, cells were seeded in 24-well plates at 2,000 cells/well in medium 199 supplemented as above with the exception of HBGF-1. The indicated amounts of wild-type or mutant HBGF-1 and heparin were added to the wells. The media was changed every other day. After 7 d in culture, cells were trypsinized and counted using a hemocytometer.

Competition for Binding and Cross-Linking to Cell Surface Receptors

Bovine brain–derived HBGF-1 (4) was labeled with ^{125}I using immobilized lactoperoxidase and biologically active, labeled protein was isolated using heparin-Sepharose as described (16). Confluent NIH 3T3 cells in 24-well plates were serum starved for 24 h before binding experiments in DME containing 0.5% calf serum. The cells were washed and incubated with DME containing 5 U/ml heparin, 0.5% BSA, and 25 mM Hepes, pH 7.2 (binding buffer) at room temperature for 20 min. The cells then were incubated with ^{125}I-HBGF-1 and unlabeled wild-type or mutant HBGF-1 in the presence of 5 U/ml heparin as indicated in the figure legend. The cells were incubated on ice for 90 min. The plates were aspirated and washed four times with binding buffer. The cells were then incubated for 20 min at 4°C with 1 ml of 0.3 mM disuccinimidyl suberate in PBS. The cross-linker was then aspirated off and the reaction quenched by adding 2.0 M Tris-HCl, pH 8.0. The cells were washed with PBS, scraped from the plates and pelleted for 10 s at 15,000 *g*. The pellets were extracted with 100 μl of 50 mM Tris, 1 mM EDTA, 200 mM NaCl, 1.0% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.5 for 20 min at 4°C. The extracts were centrifuged for 10 min at 15,000 *g*. The supernatants were removed and mixed with an equal volume of Laemmli sample buffer for SDS-PAGE analysis.

Stimulation of Protein Tyrosine Kinase Activity

NIH 3T3 cells were grown to confluence in 100 mm dishes and serum starved as described above. The cells were then exposed to diluent 1.0, or 10 ng/ml of wild-type or mutant HBGF-1 for 10 min at 37°C. The cells were washed once with cold PBS then lysed in buffer containing 10 mM Tris, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 1.0% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, pH 7.4. The cells were scraped from the plates, vortexed, and incubated on ice for 10 min. Lysates were clarified by centrifugation at 10,000 *g* for 10 min at 4°C and the supernatants were mixed with an equal volume of 2× Laemmli sample buffer. Samples (normalized to cell number) were subjected to PAGE in the presence of SDS. The proteins were transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine antibodies as described (15). The blots were incubated with ^{125}I-protein A and phosphotyrosine-containing proteins were visualized by autoradiography. In some experiments the initial cell lysates were incubated with a pre-bound anti–phospholipase C-γ antibody/protein A–Sepharose complex (31) for 90 min at 4°C. The beads were washed with 20 mM Hepes, 0.1% Triton X-100, 150 mM NaCl, 10% glycerol, pH 7.5. Immunoprecipitated proteins were eluted from the beads with 2× Laemmli sample buffer and subjected to PAGE and Western blotting with anti-phosphotyrosine antibodies as described above.

RNA Gel Blot Analysis

NIH 3T3 cells were incubated for 48 h in DME/0.5% FCS and then either left unstimulated or stimulated with wild-type or mutant HBGF-1 for the indicated times. Cells were harvested, total RNA was prepared (17), and 10 μg of each sample was separated by electrophoresis on 1.2% agarose gels containing formaldehyde. The gels were stained with ethidium bromide photographed to verify that each lane contained an equal amount of undegraded ribosomal RNA. RNA was electroblotted onto Zetabind nylon filters and cross-linked by UV irradiation. The restriction fragments used and source of the DNA probes were as follows: (*a*) c-*fos*, 2.8-kb Nco I/Xho I fragment of pc-*fos*-1; American Type Culture Collection, Rockville, MD; (*b*) c-*jun*, 1.5-kb Hind III/Bam HI fragment of ph-cJ-1; gift of P. Angel, University of California, La Jolla, CA; (*c*) c-*myc*, 1.4-kb Sst I fragment of pHSR-1; ATCC; (*d*) glyceraldehyde 3-phosphate dehydrogenase, 0.5-kb Pst I/Xba I fragment of pHcGAP; ATCC. The probes were labeled with [^{32}P]dCTP (3,000 Ci/mmol) using a random primer labeling kit. Hybridization and filter washes were as described (17). Blots were exposed to Kodak XAR5 film at −70°C.

Transfection of NIH 3T3 Cells with HBGF-1 Eukaryotic Expression Plasmids

NIH 3T3 cells in 100 mm dishes were transfected with plasmid DNA by the calcium phosphate precipitation method (44). Cells were incubated with either 1 μg of pSV2 neo (41) or co-transfected with a mixture (1:10 μg) of pSV2 neo and either HBGF-1 wild-type expression vector (p267) or HBGF-1 mutant expression vector (p268). The plasmid p267 is described in Jaye et

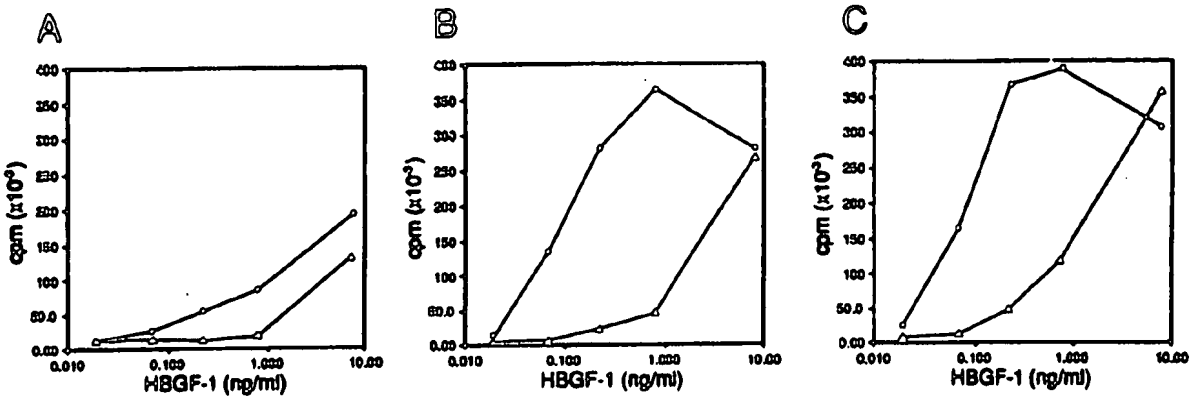

Figure 1. Stimulation of DNA synthesis in NIH 3T3 cells by wild-type and mutant HBGF-1. Cells were grown to near confluence and serum starved for 24 h as described in Materials and Methods. Cells were treated with the indicated concentrations of wild-type (○) or mutant (△) HBGF-1, incubated for 18 h, and then pulsed with 0.5 μCi of [³H]thymidine/ml for 4 h. The cells were harvested and incorporation of radioactivity was determined. Both wild-type and mutant HBGF-1 were assayed in the presence of 0 (*A*), 5 (*B*), or 50 U/ml heparin (*C*).

al. (23); p268 was constructed by replacing the 297bt Pvu II/Bgl II fragment of p267 (encoding amino acids 38–155) with the corresponding region from the prokaryotic expression plasmid pEI32 using standard subcloning methods. Cells were split to 10 dishes and transfected colonies were selected by incubating the cells in DME, 10% calf serum containing 500 μg/ml Geneticin. The media was changed every 3–4 d. After 4 wk, transfected colonies were analyzed for HBGF-1 expression by Western blot analysis using rabbit polyclonal HBGF-1–specific antibodies and ^{125}I-protein A as described above.

Results

Heparin-binding Properties of HBGF-1 Mutant pI32E

A drastic reduction in the apparent affinity of HBGF-1 containing glutamic acid in place of lysine at position 132 was observed during the purification of the recombinant proteins from the *Escherichia coli* lysates. Recombinant wild-type HBGF-1 from *E. coli* lysates can be purified to near homogeneity with a single heparin-Sepharose step. The protein binds the immobilized heparin during extensive washing with 0.5 and 0.65 M NaCl–containing buffers and is eluted with a single step of 1.5 M NaCl–containing buffer. In contrast, heparin-Sepharose affinity-based chromatography could not be used as a single purification step for the mutant HBGF-1. The mutant protein binds immobilized heparin in the presence of 0.1 M NaCl but was eluted during the 0.5 M NaCl wash. Both wild-type and mutant HBGF-1 (1.5 and 0.5 M NaCl eluates, respectively) could be purified to apparent homogeneity using reversed-phase HPLC. Detailed analysis of the apparent affinities of the two purified proteins for immobilized heparin-Sepharose using relatively shallow, linear NaCl gradients indicated that the mutant HBGF-1 eluted with 0.45 M NaCl whereas wild-type required 1.1 M NaCl to be eluted (data not shown). For all of the assays described below we used reversed-phase HPLC purified wild-type or mutant HBGF-1. Protein concentrations were determined by amino acid analysis of preparations that had been shown to be the desired HBGF-1 form by peptide mapping and amino acid sequence analysis (data not shown).

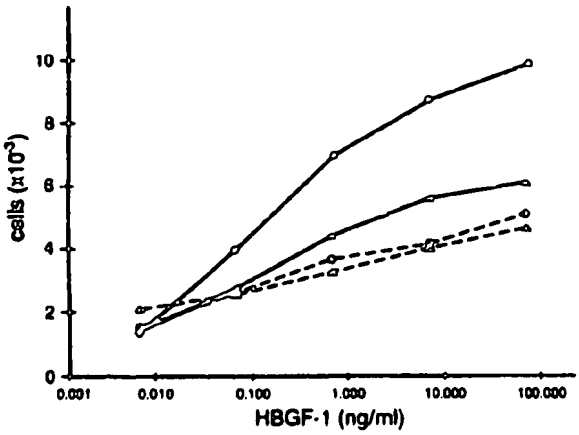

Figure 2. Ability of wild-type and mutant HBGF-1 to stimulate growth of human umbilical vein endothelial cells. Cells were seeded and cultured as described in Materials and Methods. Cell number after 7 d in culture in the presence of the indicated concentrations of wild-type (○/○) or mutant (△/△) HBGF-1 in the absence (○/△) or presence (○/△) of 50 U/ml heparin is shown.

Mitogenic Properties of HBGF-1 Mutant pI32E

The ability of the HBGF-1 mutant to stimulate mitogenesis was compared to that of the wild-type protein using two different assays. In the first, the ability of the two proteins to stimulate DNA synthesis in NIH 3T3 cells as measured by [³H]thymidine incorporation was examined. The assays were conducted over a broad range of HBGF-1 and heparin concentrations. Two important points can be made from the data in Fig. 1. One, the wild-type HBGF-1 has a dramatic requirement for the presence of heparin for optimal mitogenic activity and, two, the mutant HBGF-1 is significantly less potent than wild-type protein in the presence of added heparin. As can be seen in Fig. 1, the maximal difference in mitogenic potency was observed in the presence of 5 U/ml heparin (~30-fold). Little difference (approximately three-

Table I. Cell Number (× 10^{-3})

	Growth factor concentration (ng/ml)					
	0	0.1	0.5	1	5	10
GLU_{132} HBGF-1	1.6	1.6	1.3	1.2	1.7	1.4
Wild-type HBGF-1	1.7	2.0	1.9	2.9	12.6	16.6

fold) between the wild-type and mutant protein is seen in the absence of added heparin because of the relative lack of mitogenic activity of wild-type human recombinant HBGF-1 in the absence of heparin. The possibility that the reduced mitogenic activity of the mutant HBGF-1 is related directly to its reduced apparent affinity for immobilized heparin is supported by the observation that the difference in the mitogenic potency between the wild-type and mutant protein is reduced to ~18-fold in the presence of 50 U/ml heparin.

In the second mitogenesis assay the abilities of the wild-type and mutant proteins to support the proliferation of human umbilical vein endothelial cells were compared. The results shown in Fig. 2 are consistent with those described above in that they demonstrate a dramatic heparin requirement of the wild-type HBGF-1 for biological activity and that the mutant HBGF-1 is not able to support cell proliferation to the same extent as the wild-type protein. These experiments were conducted in the presence of 50 U/ml heparin and the endothelial cells were seeded in the presence of 10 ng/ml wild-type HBGF-1. When growth assays were conducted in the presence of 5 U/ml heparin without wild-type protein during the seeding, mitogenic deficiencies of the mutant protein were more pronounced (Table I). The results shown in Fig. 3 demonstrate that the reduced mitogenic activity of the mutant HBGF-1 does not appear to be the result of any increased susceptibility of the protein to proteolytic digestion by components in serum or the conditioned media of NIH 3T3 cells.

Receptor-binding Activity of HBGF-1 Mutant p132E

The results presented above are consistent with the observations of Harper and Lobb (19) using bovine brain-derived HBGF-1 selectively methylated at lysine 132, although the magnitude of the reduction in mitogenic potency (~30-fold for 3T3 cell assay) as compared with the ~4-fold decrease reported by Harper and Lobb (19) is significantly greater. They also reported reduced receptor-binding activity for the modified protein. We examined the abilities of the wild-type and mutant recombinant HBGF-1 to compete with ^{125}I-labeled bovine HBGF-1 for binding to cell surface receptors on NIH 3T3 cells at a concentration of added heparin (5 U/ml) where the difference in mitogenic potencies of the two proteins was greatest.

The receptor-binding activity of the mutant HBGF-1 was established by competition for cross-linking of ^{125}I-HBGF-1 to 150,000- and 130,000-*M*, proteins present on the surface of NIH 3T3 cells (16). The results shown in Fig. 4 demonstrate that the mutant HBGF-1 is similar to wild-type protein in its ability to compete for receptor–ligand cross-linking.

The functional consequences of HBGF-1 binding to its cell surface receptor include stimulation of protein tyrosine kinase activity (8, 15, 20) including phosphorylation of phospholipase C-γ (6). Fig. 5 *A* demonstrates that both wild-type and mutant HBGF-1 are able to increase the phosphotyrosine content of 150,000-, 90,000-, and 70,000-*M*, proteins and, to a lesser extent, proteins with lower relative molecular masses as judged by Western blot analysis with phosphotyrosine-specific antibodies. The dose response and extent of activation is similar for the two forms of the growth factor. Stimulation of the phosphotyrosine content of phospholipase C-γ was examined by anti-phosphotyrosine Western blot analysis of 3T3 cell lysates after immunoprecipitation using antibodies that recognize phospholipase C-γ. Fig. 5 *B* demonstrates that mutant HBGF-1 shares with wild-type HBGF-1 the ability to stimulate tyrosine phosphorylation of phospholipase C-γ. These data regarding stimulation of tyrosine kinase activity by wild-type and mutant HBGF-1 are in good agreement with the receptor-binding data described above but do not provide insight into the functional basis for the relatively poor mitogenic capacity of this HBGF-1 mutant.

Protooncogene Induction by Wild-Type and Mutant HBGF-1

The results described above indicate that the functional properties of the mutant HBGF-1 associated with events that occur at the cell surface (i.e., receptor-binding and tyrosine kinase activation) are normal with respect to those of wild-type HBGF-1. In addition to tyrosine kinase activation, another early response to HBGF-1 receptor-binding is the elevation of protooncogene mRNA levels (17). To determine the effect of wild-type and mutant HBGF-1 on protooncogene expression, NIH 3T3 cells were serum starved and then either left unstimulated or stimulated with 10 ng/ml wild-type or mutant HBGF-1. Heparin (5 U/ml) was also added to the cells receiving growth factor. Cells were collected at various times after stimulation, RNA was prepared, and levels of c-*fos*, c-*jun*, c-*myc*, and glyceraldehyde 3-phosphate dehydrogenase mRNA (as a control for the amount of RNA loaded in each lane) were assayed by RNA gel blot analysis. Wild-type and mutant HBGF-1 increased protooncogene mRNA levels to a similar degree; maximal levels were observed at 30 min (c-*fos*, c-*jun*) or 2 h (c-*myc*) after stimulation (Fig.

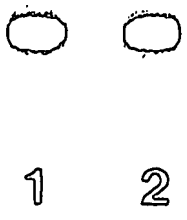

Figure 3. Analysis of the relative stability of wild-type and mutant HBGF-1 in NIH 3T3 cell-conditioned media. The wild-type and mutant proteins were labeled and purified as described in Materials and Methods. The proteins were incubated in the presence of NIH 3T3 cell-conditioned media for 48 h at 37°C and then subjected to SDS-PAGE. The gels were dried and labeled proteins visualized by autoradiography. Lane 1 contains wild-type HBGF-1 and lane 2 mutant HBGF-1. The apparent molecular weights of both proteins are identical to that of HBGF-1 before incubation.

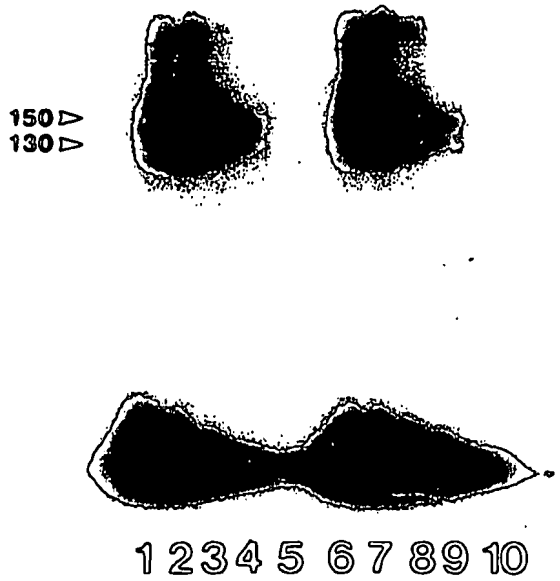

Figure 4. Ability of wild-type and mutant HBGF-1 to compete with ^{125}I-labeled bovine HBGF-1 for cross-linking to 150,000- and 130,000-mol wt cell surface receptors. NIH 3T3 cells were incubated with 1 ng/ml bovine ^{125}I-HBGF-1 and either 0.5, 1.0, 5.0, 10.0, or 50.0 ng/ml of wild-type (lanes 1–5) or mutant (lanes 6–10) human recombinant HBGF-1 in the presence of 5 U/ml heparin. After incubation, the cells were treated with cross-linking reagents as described in Materials and Methods. The apparent molecular weights of cross-linked species were determined after SDS-PAGE and autoradiography. The positions of two cross-linked 150,000- and 130,000-mol wt species, which correspond to the known apparent molecular weights of HBGF receptors, are indicated with arrows.

6). The addition of heparin alone did not induce protooncogene expression. Since the mitogenic differences between the wild-type and mutant HBGF-1 are more pronounced at lower growth factor concentrations, we also stimulated cells with 0.5, 1.0, 5.0, and 10 ng/ml wild-type and mutant growth factor (again in the presence of heparin). At all four concentrations used, the wild-type and mutant HBGF-1 were similar in their ability to induce c-*fos* mRNA expression (Fig. 7).

Overexpression of Wild-Type and Mutant HBGF-1 in Transfected NIH 3T3 Cells

It was demonstrated previously that overexpression of wild-type HBGF-1 in transfected Swiss 3T3 cells resulted in cells with an elongated, transformed morphological phenotype that grew to higher saturation densities (23). This transformed phenotype occurred even though the HBGF-1 polypeptide was not detectable in the conditioned media of these cells. We have shown that the mutant HBGF-1 is not a potent mitogen although it can bind receptor and initiate early events associated with mitogenic signal transduction. To investigate whether the intracellular function of the mutant HBGF-1 was altered, we examined the ability of this protein to induce a transformed phenotype in NIH 3T3 cells. Cells were either transfected with a plasmid conferring neomycin resistance or co-transfected with the neomycin resistance plasmid and wild-type or mutant HBGF-1 expression vectors.

Fig. 8 shows the results of Western blot analysis of transfected cell lysates using HBGF-1–specific antibodies. The Western blot analysis was normalized to cell number and provides the basis for our designation of relatively high or low levels of HBGF-1 expression. The results shown in Fig. 9 demonstrate that cells expressing a high level of wild-type HBGF-1 (Fig. 9 *B*) and to some extent a low level of wild-

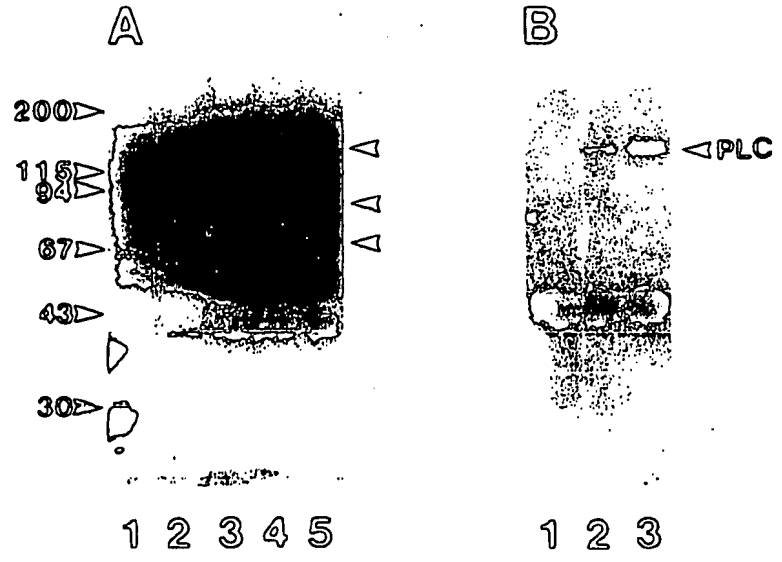

Figure 5. Stimulation of protein tyrosine kinase activity by wild-type and mutant HBGF-1. (*A*) Serum starved NIH 3T3 cells were either (lane *1*) unstimulated or treated with 5 U/ml heparin and (lane *2*) 1 ng/ml wild-type; (lane *3*) 10 ng/ml wild-type; (lane *4*) 1 ng/ml mutant; or (lane *5*) 10 ng/ml mutant HBGF-1. The cells were processed as described in Materials and Methods and phosphotyrosine-containing proteins were visualized using antiphosphotyrosine antibodies and ^{125}I-protein A. The arrows indicate the positions of 150,000-, 90,000-, and 70,000-mol wt proteins whose phosphotyrosine content are increased by the addition of wild-type or mutant HBGF-1. (*B*) Cells were incubated as in *A* with the exception that cell lysates were immunoprecipitated with anti-phospholipase C-γ antibodies before Western blot analysis with anti-phosphotyrosine antibodies. Cells were either (lane *1*) unstimulated or treated with (lane 2) 10 ng/ml wild-type, or (lane *3*) 10 ng/ml mutant HBGF-1. The arrow shows the position of a 150,000-mol wt protein whose phosphotyrosine content is increased by treatment with wild-type or mutant HBGF-1.

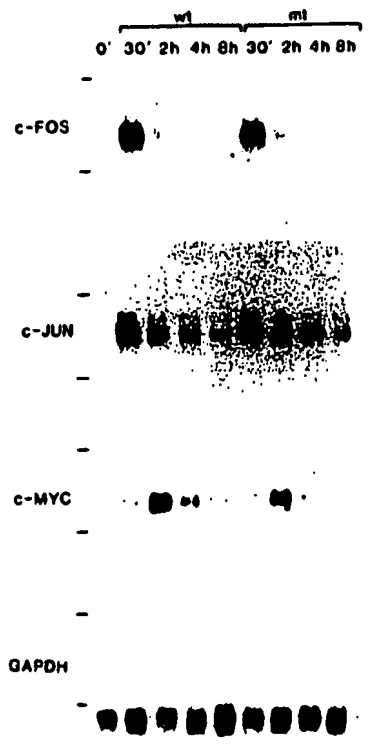

Figure 6. Effect of wild-type and mutant HBGF-1 on protooncogene mRNA levels. Serum starved NIH 3T3 cells were either left unstimulated or treated with heparin (5 U/ml) and 10 ng/ml wild-type (*wt*) or mutant (*mt*) HBGF-1 for the indicated time periods. RNA was prepared and used for RNA gel blot hybridization using the radiolabeled DNA probes indicated on the left side (*GAPDH*, glyceraldehyde 3-phosphate dehydrogenase). The upper and lower tick marks on the left side of each panel represent the positions of 28 and 18S rRNA, respectively.

type HBGF-1 (Fig. 9 *D*) have acquired a more polar, elongated phenotype characteristic of transformed 3T3 cells. This phenotype is not seen in cells expressing neomycin resistance alone (Fig. 9 *A*) or in cells expressing relatively high levels of mutant HBGF-1 (Fig. 9 *C*). It should be noted that we have not been able to detect HBGF-1 immunoreactivity in the media conditioned by these cells and that the cells expressing relatively high levels of wild-type HBGF-1 show enhanced growth in soft agar relative to untransfected cells or cells expressing high levels of the mutant HBGF-1 (data not shown). These results are consistent with the results of the mitogenic assays described above which demonstrate that the growth-promoting activity of the mutant HBGF-1 is relatively low when compared to the wild-type protein.

Discussion

The experiments described in this report were initiated as a result of the chemical modification studies of HBGF-1 reported by Harper and Lobb (19). They demonstrated that reductive methylation of HBGF-1 resulted in selective, stoichiometric modification of lysine residue 132 (using the 1–154 numbering system for full-length HBGF-1). It was suggested that modification of this residue, which is conserved in all HBGF-1 and HBGF-2 sequences reported to date, was responsible for the reduced apparent affinity for immobilized heparin, the reduced mitogenic capacity, and the reduced receptor-binding activity of the modified protein. The results presented here using site-directed mutagenesis to address the role of lysine 132 on the functional properties of HBGF-1 are in general agreement with the conclusions of Harper and Lobb (19). Specifically, substitution of lysine 132 for glutamic acid reduces the apparent affinity of the recombinant protein for immobilized heparin (elutes at 0.45 M NaCl compared with 1.1 M NaCl for wild-type) and significantly reduces the mitogenic potency of the growth factor. The reduced mitogenic potency may be a direct consequence of the reduced apparent affinity of the mutant HBGF-1 for heparin since it has been demonstrated that the class 1 heparin-binding growth factors in general (29) and human HBGF-1 in particular (22, 43) are dependent on the presence of heparin for optimal biological activity.

Our results do not support the notion that the reduced mitogenic capacity of HBGF-1 containing glutamic acid in place of lysine at position 132 is due to reduced binding to cell surface receptors. The receptor-binding properties of the mutant HBGF-1 are not distinguishable from those of the wild-type protein as judged by cross-linking experiments (see Fig. 4). In addition, the mutant HBGF-1 is able to induce the same pattern of tyrosine kinase phosphorylation as is the wild-type protein (see Fig. 5) and can induce protooncogene expression (see Fig. 6). The majority of the studies presented here utilize a heparin concentration of 5 U/ml; the concentration where maximal difference between the mitogenic activity of wild-type and mutant HBGF-1 was observed in the 3T3 cell thymidine incorporation assay. It should be noted that in the absence of heparin, the mutant HBGF-1 competes poorly with labeled wild-type HBGF-1 in cross-

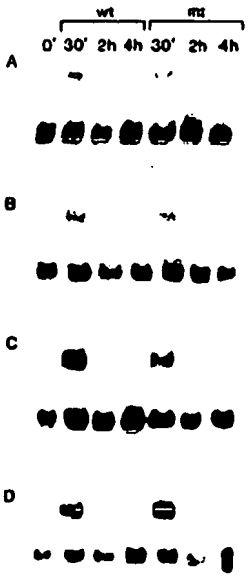

Figure 7. Effect of different concentrations of wild-type and mutant HBGF-1 on c-*fos* mRNA levels. Serum starved NIH 3T3 cells were either left unstimulated or treated with heparin (5 U/ml) and (*A*) 0.5 ng/ml, (*B*) 1.0 ng/ml, (*C*) 5.0 ng/ml, (*D*) 10 ng/ml wild-type (*wt*) or mutant (*mt*) HBGF-1 for the indicated time periods. RNA was prepared and used for RNA gel blot hybridization using the c-*fos* DNA probe (*upper panels*) or glyceraldehyde 3-phosphate dehydrogenase DNA probe (*lower panels*).

Figure 8. Western blot analysis of HBGF-1 in NIH 3T3 cells transfected with wild-type or mutant HBGF-1 expression plasmids. NIH 3T3 cells were transfected as described in Materials and Methods. The figure shows the relative levels of HBGF-1 immunoreactivity present in lysates of cells transfected with wild-type HBGF-1 (lane *1*, clone producing relatively high level of HBGF-1; lane *3*, clone producing relatively low level of HBGF-1) normal NIH 3T3 cells (lane *2*), cells transfected with pSV2neo alone (lane *4*), and cells transfected with mutant HBGF-1 (lane *5*). For each cell type, 10^5 cells were lysed with 1 ml of 2× Laemmli sample buffer and a 60-μl aliquot was used in the Western blot.

linking assays (data not shown). In addition, whereas the apparent affinity of the mutant HBGF-1 for immobilized heparin is reduced, it does bind at ionic strengths (i.e., ~0.5 M NaCl) that exceed those known to be physiologic. Thus, the data presented here indicate that the mutant can utilize the presence of heparin to restore some (i.e., receptor-binding, tyrosine kinase activation, and protooncogene induction) but not all (i.e., stimulation of [^{3}H]thymidine incorporation into DNA and endothelial cell proliferation) of the activities of the wild-type protein. Similarly, it is of interest that the wild-type protein competes with labeled HBGF-1 for receptor-binding and induces protooncogene expression at similar concentrations in the presence or absence of added heparin yet it requires added heparin in order to promote DNA synthesis and cell proliferation (Figs. 1, 2, 4, and 6; and data not shown). Thus, the relatively poor mitogenic activity of the mutant protein may be related to its reduced apparent affinity for heparin. The data presented here demonstrate that "high" affinity receptor-binding, activation of tyrosine kinase activity, tyrosine phosphorylation of specific substrates, and induction of protooncogene expression may be necessary but are not, by themselves, sufficient to sustain a mitogenic response to the presence of HBGF-1. These results are consistent with the observations of Escobedo and Williams (12) who showed by site-directed mutagenesis of the PDGF receptor and cDNA transfection that mutants could be constructed that were responsive to PDGF with respect to receptor tyrosine kinase activation and increased phosphatidylinositol turnover but did not elicit a mitogenic re-

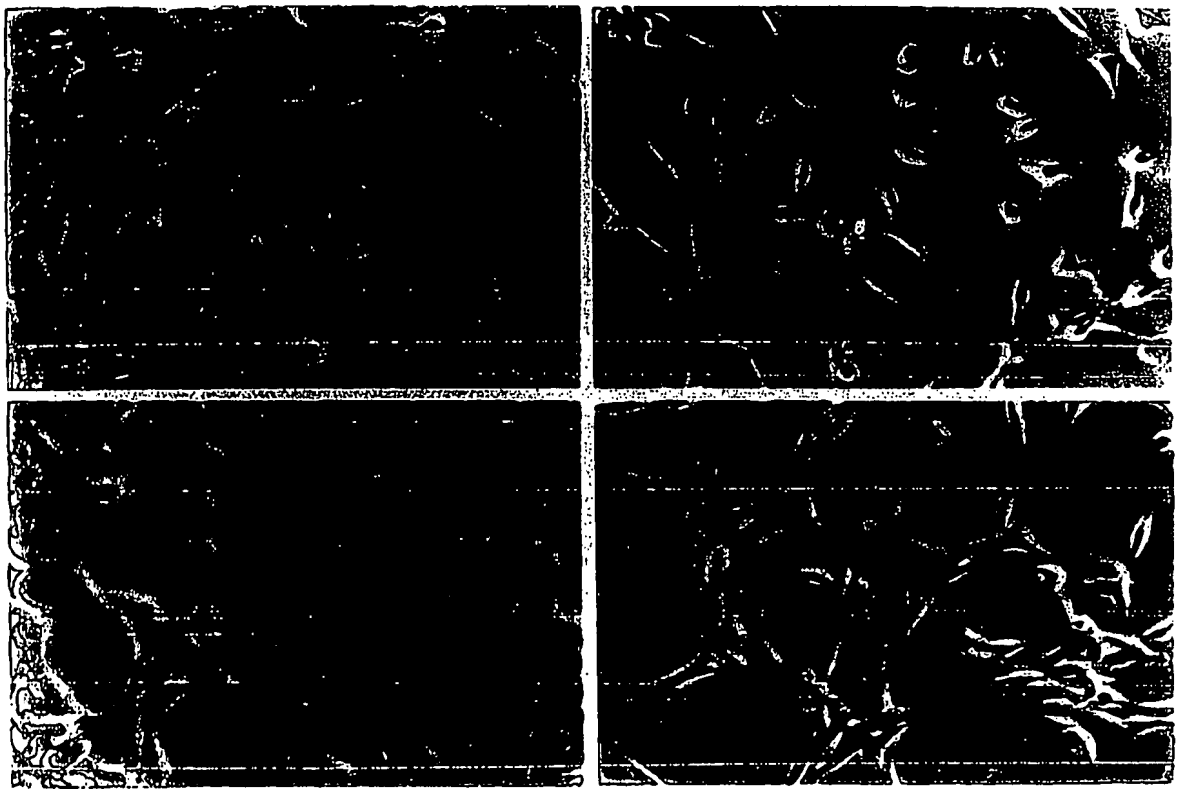

Figure 9. Morphology of NIH 3T3 cells transfected with wild-type or mutant HBGF-1 expression plasmids. The figure shows micrographs of the same NIH 3T3 cells analyzed by Western blot analysis in Fig. 8. *A* shows cells transfected with pSV2neo only and *B–D* show cells co-transfected with pSV2neo and expression vectors for wild-type (*B* and *D*) and mutant (*C*) HBGF-1. The cells shown in *B* correspond to those expressing relatively high levels of HBGF-1 (Fig. 8, lane *1*), whereas those shown in *D* correspond to those expressing relatively little HBGF-1 (Fig. 8, lane *3*).

sponse to PDGF. Similarly, Severinsson et al. (40) used similar methods to generate a system where the mutant receptor could mediate an increase in *c-fos* expression in response to PDGF but not actin reorganization or mitogenesis.

The mitogenic deficiencies of the mutant HBGF-1 may be due to reduced biological stability in tissue culture medium, reduced binding to cell surface proteoglycans, an altered intracellular stability, and/or an altered affinity for an intracellular receptor or binding protein. It has been established that the presence of heparin protects HBGF-1 from thermal and proteolytic inactivation (28, 37). In addition, it has been shown that ^{125}I-labeled HBGF-1 is relatively insensitive to lysosomal degradation after receptor-mediated endocytosis (14). There is no obvious difference in the susceptibility of wild-type and mutant HBGF-1 to proteolytic cleavage by the conditioned media of NIH 3T3 cells cultured in the presence of 10% calf serum. However, the relative resistance of wild-type and mutant HBGF-1 to proteolytic modification in the presence of target cells or after receptor-mediated endocytosis has not been established. It is also possible that the mutant protein is more susceptible than the wild type to nonproteolytic inactivation. Further studies should reveal whether the altered activities of the mutant HBGF-1 are a consequence of its reduced apparent affinity for heparin.

In summary, the data presented here demonstrate that the various functions of HBGF-1 can be dissociated at the structural level. The observation that site-directed mutagenesis can be used to produce recombinant proteins with "normal" receptor-binding activity and reduced mitogenic activity indicates that similar methods could be used to produce potent antagonists of HBGF-1. More importantly, these results indicate that it may be possible through structure–function analysis and site-directed mutagenesis to generate mutants that retain certain (i.e., chemotactic, mitogenic, or heparin-binding) but not other biological functions characteristic of the wild-type protein. Finally, whereas the data presented on the receptor-binding and tyrosine kinase activation properties of the p132E mutant demonstrate that a lysine residue at this position is not critical for these functions, it is still possible that methylation of a lysine at this position could lead to reduced receptor-binding activity of HBGF-1 (19).

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References

1. Baird, A., D. Schubert, N. Ling, and R. Guillemin. 1986. Receptor- and heparin-binding domains of basic fibroblast growth factor. *Proc. Natl. Acad. Sci. USA.* 85:2324–2328.
2. Bouche, G., N. Gas, H. Prats, V. Baldin, J.-P. Tauber, J. Teissié, and F. Amalric. 1987. Basic fibroblast growth factor enters the nucleolus and stimulates the transcription of ribosomal genes in ABAE cells undergoing G_0→G_1 transition. *Proc. Natl. Acad. Sci. USA.* 84:6770–6774.
3. Burgess, W. H., and T. Maciag. 1989. The heparin-binding (fibroblast) growth factor family of proteins. *Annu. Rev. Biochem.* 58:575–606.
4. Burgess, W. H., T. Mehlman, R. Friesel, W. V. Johnson, and T. Maciag. 1985. Multiple forms of endothelial cell growth factor: rapid isolation and biological and chemical characterization. *J. Biol. Chem.* 260:11389–11392.
5. Burgess, W. H., T. Mehlman, D. R. Marshak, R. A. Fraser, and T. Maciag. 1986. Structural evidence for endothelial cell growth factor β is the precursor of both endothelial cell growth factor α and cell surface fibroblast growth factor. *Proc. Natl. Acad. Sci. USA.* 83:7216–7220.
6. Burgess, W. H., C. A. Dionne, U. Kaplow, R. Mudd, R. Friesel, A. Zilberstein, J. Schlessinger, and M. Jaye. 1990. "Characterization and cDNA cloning of phosphatidylinositol-(4,5)-bisphosphate phosphodiesterase cleavage enhancing factor 1 (acidic fibroblast growth factor)-activated tyrosine kinase. *Mol. Cell Biol.* 10:4770–4777.
7. Casey, G., R. Smith, D. McGillivray, G. Peters, and C. Dickson. 1986. Characterization and chromosome assignment of the human homolog of int-2, a potential proto-oncogene. *Mol. Cell. Biol.* 6:502–510.
8. Coughlin, S. R., J. Barr, L. S. Courtneidge, L. T. Williams, and L. T. Williams. 1988. Acidic and basic fibroblast growth factors stimulate tyrosine kinase activity *in vivo*. *J. Biol. Chem.* 263:988–993.
9. Dalla Favera, P., and C. Basilico. 1987. Isolation of a rearranged human transforming gene following transfection of Kaposi sarcoma DNA. *Proc. Natl. Acad. Sci. USA.* 84:5660–5664.
10. Dickson, C., and G. Peters. 1987. Potential oncogene product related to growth factors. *Nature (Lond.).* 326:833–836.
11. Dickson, C., R. Smith, S. Brookes, and G. Peters. 1984. Tumorigenesis by mouse mammary tumor virus: proviral activation of a cellular gene in the common integration region int-2. *Cell.* 37:529–536.
12. Escobedo, J. A., and L. T. Williams. 1988. A PDGF receptor domain essential for mitogenesis but not for many other responses to PDGF. *Nature (Lond.).* 335:85–87.
13. Finch, P. W., J. S. Rubin, T. Miki, D. Ron, and S. Aaronson. 1989. Human KGF is FGF-related with properties of a paracrine effector of epithelial cell growth. *Science (Wash. DC).* 245:752–755.
14. Friesel, R., and T. Maciag. 1988. Internalization and degradation of heparin-binding growth factor-1 by endothelial cells. *Biochem. Biophys. Res. Commun.* 151:957–964.
15. Friesel, R., W. H. Burgess, and T. Maciag. 1989. Heparin-binding growth factor 1 stimulates tyrosine phosphorylation in NIH 3T3 cells. *Mol. Cell Biol.* 9:1857–1865.
16. Friesel, R., W. H. Burgess, T. Mehlman, and T. Maciag. 1986. The characterization of the receptor for endothelial cell growth factor by covalent ligand attachment. *J. Biol. Chem.* 261:7581–7584.
17. Gay, C. G., and J. A. Winkles. 1990. Heparin-binding growth factor-1 stimulation of human endothelial cells induces platelet-derived growth factor A-chain gene expression. *J. Biol. Chem.* 265:3284–3292.
18. Gospodarowicz, D., G. Neufeld, and L. Schweigerer. 1987. Fibroblast growth factor: structural and biological properties. *J. Cell. Physiol. Suppl.* 5:15–26.
19. Harper, J. W., and R. R. Lobb. 1988. Reductive methylation of lysine residues in acidic fibroblast growth factor: effect on mitogenic activity and heparin affinity. *Biochemistry.* 27:671–678.
20. Huang, S. S., and J. S. Huang. 1986. Association of bovine brain-derived growth factor receptor with protein tyrosine kinase activity. *J. Biol. Chem.* 261:9568–9571.
21. Jaye, M., R. Howk, W. Burgess, G. A. Ricca, I. Chiu, M. W. Ravera, S. J. O'Brien, W. S. Modi, T. Maciag, and W. N. Drohan. 1986. Human endothelial cell growth factor: cloning, nucleotide sequence, and chromosome localization. *Science (Wash. DC).* 233:541–545.
22. Jaye, M., W. H. Burgess, A. B. Shaw, and W. N. Drohan. 1987. Biological equivalence of natural bovine and recombinant human α-endothelial cell growth factor. *J. Biol. Chem.* 262:16612–16617.
23. Jaye, M., R. M. Lyall, R. Mudd, J. Schlessinger, and N. Sarver. 1988. Expression of acidic fibroblast growth factor cDNA confers growth advantage and tumorigenesis to Swiss 3T3 cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:963–969.
24. Kan, M., D. DiSorbo, J. Hou, H. Hoshi, P. E. Monsson, and W. L. McKeehan. 1988. High and low affinity binding of heparin-binding growth factor to a 130-kDa receptor correlates with stimulation and inhibition of growth of a differentiated human hepatoma cell. *J. Biol. Chem.* 263:11305–11313.
25. Kodo, T., A. Sasaki, S. Matsushima, and M. Kobinoma. 1987. A transforming gene, hst, found in NIH 3T3 cells transformed with DNA from stomach cancers and a colon cancer. *Jpn. J. Cancer Res.* 78:325–328.
26. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 277:680–685.
27. Lobb, R. R. 1988. Clinical applications of heparin-binding growth factors. *Eur. J. Clin. Invest.* 18:321–336.
28. Lobb, R. R. 1988. Thrombin inactivates acidic fibroblast growth factor but not basic fibroblast growth factor. *Biochemistry.* 27:2572–2578.
29. Lobb, R. R., J. W. Harper, and J. W. Fett. 1986. Purification of heparin-binding growth factors. *Anal. Biochem.* 154:1–14.
30. Deleted in proof.
31. Margolis, B., S. G. Rhee, S. Felder, M. Mervic, R. Lyall, A. Levitzki, A. Ullrich, A. Zilberstein, and J. Schlessinger. 1989. EGF induces tyrosine phosphorylation of phospholipase C-γ: a potential mechanism for

EGF receptor signaling. *Cell.* 57:1101–1107.
32. Marics, L., J. Adelaide, F. Raybaud, M.-O. Mattei, F. Coulier, J. Planche, O. Bascaules, and D. Birnbaum. 1989. Characterization of the HST-related FGF-6 gene, a new member of the fibroblast growth factor gene family. *Oncogene.* 4:335–340.
33. Mehlman, T., and W. H. Burgess. 1990. Identification and characterization of heparin-binding proteins using a gel overlay procedure. *Anal. Biochem.* 188:199–163.
34. Moscatelli, D. 1988. Metabolism of receptor-bound and matrix-bound basic fibroblast growth factor by bovine capillary endothelial cells. *J. Cell Biol.* 107:753–759.
35. Rogelj, S., W. Quarto, T. Morimoto, and D. B. Riffin. 1990. Nuclear and cytoplasmic localization of different basic fibroblast growth factor species. *J. Cell. Physiol.* 140:102–110.
36. Riffin, D. B., and D. Moscatelli. 1989. Recent developments in the cell biology of basic fibroblast growth factor. *J. Cell. Biol.* 109:1–6.
37. Saksela, T. K., W. Johnson, R. Friesel, R. Clark, and T. Maciag. 1988. Heparin protects heparin-binding growth factor-1 from proteolytic inactivation *in vitro*. *Biochem. Biophys. Res. Commun.* 152:432–440.
38. Sakamoto, H., M. Mori, M. Taira, T. Yoshida, S. Matsukura, K. Shimizu, M. Terada, and T. Sugimura. 1986. Transforming gene from human stomach cancers and a noncancerous portion of stomach mucosa. *Proc. Natl. Acad. Sci. USA.* 83:3097–3101.
39. Schubert, D., N. Ling, and A. Baird. 1987. Multiple influences of a heparin-binding growth factor on neuronal development. *J. Cell Biol.* 104:635–643.
40. Severinsson, L., B. Ek, K. Mellstrom, L. Claesson-Welsh, and C.-H. Heldin. 1990. Deletion of the kinase insert sequence of the platelet-derived growth factor β-receptor affects receptor kinase activity and signal transduction. *Mol. Cell. Biol.* 10:201–209.
41. Southern, P. J., and P. Berg. 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Genet.* 1:327–341.
42. Ueno, N., A. Baird, F. Esch, N. Ling, and R. Guillemin. 1986. Isolation of an amino terminal extended form of basic fibroblast growth factor. *Biochem. Biophys. Res. Comm.* 138:580–588.
43. Ulbrich, S., D. Lagente, J. Choay, Y. Courtois, and M. Lenfant. 1986. Structure activity relationship in heparin: stimulation of non-vascular cells by a synthetic heparin pentasaccharide in cooperation with human acidic fibroblast growth factors. *Biochem. Biophys. Res. Commun.* 139:728–732.
44. Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub, and L. Chasin. 1979. DNA-mediated transfer of the adenine phosphoribosyl transferase locus into mammalian cells. *Proc. Natl. Acad. Sci. USA.* 76:1373–1376.
45. Yuasa, Y., and K. Sudo. 1987. Transforming genes in human hepatomas detected with a tumorigenicity assay. *Jpn. J. Cancer Res.* 78:1036–1040.
46. Zhan, X., A. Culpepper, M. Reddy, J. Lovelace, and M. Goldfarb. 1987. Human oncogenes detected by a defined medium culture assay. *Oncogene.* 1:369–376.
47. Zhan, X., B. Bates, X. Hu, and M. Goldfarb. 1988. The human FGF-5 oncogene encodes a novel protein related to fibroblast growth factors. *Mol. Cell. Biol.* 8:3487–3495.